Multiple forms of an inositol polyphosphate 5-phosphatase form signaling complexes with Shc and Grb2

W.M. Kavanaugh*¶, D.A. Pot*†‡¶, S.M. Chin*, M. Deuter-Reinhard*, A.B. Jefferson§, F.A. Norris§, F.R. Masiarz*, L.S. Cousens*, P.W. Majerus§ and L.T. Williams*†‡

Background: Shc and Grb2 form a complex in cells in response to growth factor stimulation and link tyrosine kinases to Ras during the resulting signaling process. Shc and Grb2 each contain domains that mediate interactions with other unidentified intracellular proteins. For example, the Shc PTB domain binds to 130 kDa and 145 kDa tyrosine-phosphorylated proteins in response to stimulation of cells by growth factors, cytokines and crosslinking of antigen receptors. The Grb2 SH3 domains bind to an unidentified 116 kDa protein in T cells. We have identified three proteins, of 110 kDa, 130 kDa and 145 kDa, as a new family of molecules encoded by the same gene. *In vivo* studies show that these proteins form signal transduction complexes with Shc and with Grb2.

Results: The 130 kDa and 145 kDa tyrosine-phosphorylated proteins that associate with the Shc PTB domain were purified by conventional chromatographic methods. Partial peptide and cDNA sequences corresponding to these proteins, termed SIP-145 and SIP-130 (SIP for signaling inositol polyphosphate 5-phosphatase), identified them as SH2 domain-containing products of a single gene and as members of the inositol polyphosphate 5-phosphatase family. The SIP-130 and SIP-145 proteins and inositol polyphosphate 5-phosphatase activity associated with Shc *in vivo* in response to B-cell activation. By using an independent approach, expression cloning, we found that the Grb2 SH3 domains bind specifically to SIP-110, a 110 kDa splice variant of SIP-145 and SIP-130, which lacks the SH2 domain. The SIP proteins hydrolyzed phosphatidylinositol (3,4,5)-trisphosphate (PtdIns (3,4,5)-P_3) and Ins (1,3,4,5)-P_4, but not PtdIns (4,5)-P_2 or Ins (1,4,5)-P_3.

Conclusions: These findings strongly implicate the inositol polyphosphate 5-phosphatases in Shc- and Grb2-mediated signal transduction. Furthermore, SIP-110, SIP-130 and SIP-145 prefer 3-phosphorylated substrates, suggesting a link to the phosphatidylinositol 3-kinase signaling pathway.

Addresses: *Chiron Corporation, Emeryville, California 94608, USA. †University of California, San Francisco, California 94143, USA. §Division of Hematology–Oncology, Departments of Internal Medicine and Biological Chemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110, USA.

‡Present address: Chiron Corporation, Emeryville, California 94608, USA.

¶The first two authors contributed equally to this work.

Correspondence to: Lewis T. Williams
E-mail address: rusty_williams@cc.chiron.com

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Background

Growth factors stimulate cell growth, differentiation and a variety of other cellular responses by activating intracellular receptor and non-receptor tyrosine kinases [1–3]. One important consequence of tyrosine phosphorylation is the creation of binding sites for proteins containing SH2 and PTB domains that specifically recognize phosphotyrosine and adjacent amino-acid residues [4]. Thus, tyrosine phosphorylation serves as a switch for the assembly of complexes of signaling proteins, within which regulatory signals are generated. In addition to the SH2 and PTB domains, the SH3 domains participate in complex assembly by binding to proline-rich target sequences present in many signaling proteins [4]. The identification of novel proteins that bind to the SH2, PTB and SH3 domains of known signaling molecules has lead to many important insights into how intracellular signaling is regulated.

Two of the most important of these proteins involved in tyrosine kinase signaling are Grb2 and Shc. Shc and Grb2 form a complex in response to growth factor stimulation or oncogenic transformation [5]. These proteins are thought to transmit mitogenic signals from receptor and non-receptor tyrosine kinases to Ras, a GTP-binding protein involved in growth regulation in normal tissues and in many human tumors [5–7]. Precisely how Shc and Grb2 influence Ras activity is unknown.

Both Shc and Grb2 contain functional domains that mediate interactions with other proteins. Grb2 contains two SH3 domains and one SH2 domain [8], whereas Shc contains one SH2 domain [9] and one PTB domain [10]. In some cases, candidate protein ligands for these domains have been identified. For example, the Grb2 SH2 domain can bind a phosphorylated tyrosine residue on Shc [5], and the Grb2 SH3 domains can bind to the

Ras nucleotide-exchange factor Sos [11–13]. However, other unidentified proteins also are found in Grb2 and Shc complexes *in vivo* [10,14]. For example, in T cells the Grb2 SH3 domains can associate with either a 116 kDa protein or with Sos [14]. Furthermore, we have previously reported that proteins of approximately 130 kDa and 145 kDa are tyrosine-phosphorylated and associate with the PTB domain of Shc in response to treatment of cells with platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), leukemia inhibitory factor or interleukin-6, and in activated T cells and B cells [10]. These observations strongly suggested that the 130 kDa and 145 kDa proteins are important signaling molecules.

We have now taken two independent approaches to identify these proteins — purification of proteins that associate with Shc, and expression cloning of proteins that bind to Grb2. Both approaches yielded molecules belonging to the inositol polyphosphate 5-phosphatase family. We found that these proteins are alternative products of the same gene and are present in different complexes in cells. Furthermore, the proteins that bind to Shc contain an SH2 domain, and the substrate preferences of these enzymes suggest a link to the phosphatidylinositol 3-kinase pathway. These findings define a new family of molecules involved in Shc- and Grb2-mediated signaling.

Results and discussion

Expression cloning of a gene encoding SIP-110

Expression cloning was used to identify proteins that interact with the SH3 domains of Grb2. The human Grb2 protein was expressed in *Escherichia coli* as a GST-fusion protein containing a consensus phosphorylation sequence for heart muscle kinase (HMK) [15]. This protein was purified, radioactively labeled using [^{32}P]ATP and HMK, and used to screen a human placental λgt11 cDNA expression library [15]. Because the probe contained both the SH2 and the SH3 domains of Grb2, we identified clones that represented autophosphorylated tyrosine kinases that were bound to the SH2 domain of Grb2. Therefore, duplicate filters were immunoblotted with anti-phosphotyrosine antibodies to identify and eliminate these SH2 domain-mediated interactions from consideration. One clone was identified that interacted with Grb2 and was not tyrosine phosphorylated. This interaction was specific for Grb2. Radioactive protein probes that contained the Vav SH3–SH2–SH3 domains, the Nck SH3–SH3–SH3 domains, or the p85 SH3 domain, did not bind to the protein encoded by this clone (data not shown).

Sequencing of the full-length 4147 base-pair (bp) clone identified an open reading frame encoding a novel protein of 976 amino acids (Fig. 1a). An in-frame stop codon 5′ to the initiator methionine codon indicated that the cDNA was complete. The predicted 110 kDa protein contained proline-rich motifs and had significant homology to a family of proteins known as inositol polyphosphate 5-phosphatases [16–20] (Fig. 1b). We have therefore named the protein SIP-110 (for signaling inositol polyphosphate 5-phosphatase, 110 kDa). A fragment of SIP-110, encoded by a partial λgt11 clone (Fig. 1a, sequences to the right of the arrow at position 2679), which contained only the carboxy-terminal proline-rich sequences, also bound specifically to Grb2, indicating that the Grb2 binding site resides in this fragment.

SIP-110 associates with the SH3 domains of Grb2 in cells

To determine whether Grb2 associates with SIP-110 *in vivo*, epitope-tagged versions of these molecules were co-expressed in COS cells. SIP-110 tagged with the hemagglutinin (HA) epitope co-immunoprecipitated with myc-tagged Grb2, and with Grb2 containing an inactivating deletion in the SH2 domain, Grb3.3 [21] (Fig. 2, lanes 3 and 7, upper panel; lane 6, lower panel). The interaction between wild-type Grb2 and SIP-110 was not detectable on immunoblots probed with the the anti-myc antibody, because Grb2 co-migrated with immunoglobulin light chain in these gels. A point mutation in either of the Grb2 SH3 domains markedly reduced or eliminated SIP-110 binding (Fig. 2, lanes 4 and 5, upper panel). Therefore, Grb2 associates with SIP-110 in cells. Furthermore, the SH3 domains, but not the SH2 domain, of Grb2 are necessary for interaction with SIP-110. Taken together, these experiments demonstrate that the SH3 domains of Grb2 bind specifically to the proline-rich region of SIP-110 in cells.

Purification of proteins that bind the Shc PTB domain

Independently, we purified proteins that associate with the PTB domain of Shc. We previously reported that proteins of approximately 145 kDa and 130 kDa are tyrosine-phosphorylated and associate with the PTB domain of Shc in response to treatment of cells with growth factors, and to antigen receptor crosslinking [10]. These observations strongly suggested that p145 and p130 are important signaling molecules. We have purified the p145 and p130 proteins from murine BAL 17 B cells stimulated by crosslinking of the B-cell antigen receptor with anti-immunoglobulin M antibodies. Affinity chromatography with anti-phosphotyrosine antibodies was followed by anion exchange chromatography and SDS–PAGE. The purification of p145 and p130 was followed at each step by assaying for proteins that specifically bound to the Shc PTB domain in a far-western blot, as described previously [10]. No proteins were found to bind to the PTB domain in crude extracts from unstimulated B cells (data not shown). In crude extracts from stimulated B cells, two proteins of approximately 130 kDa and 145 kDa bound to the PTB domain [10]. The partially purified 130 kDa and 145 kDa proteins were excised from a SDS–PAGE gel and digested with endoproteinase Lys-C. The peptides were then extracted, purified, and sequenced according to standard methods.

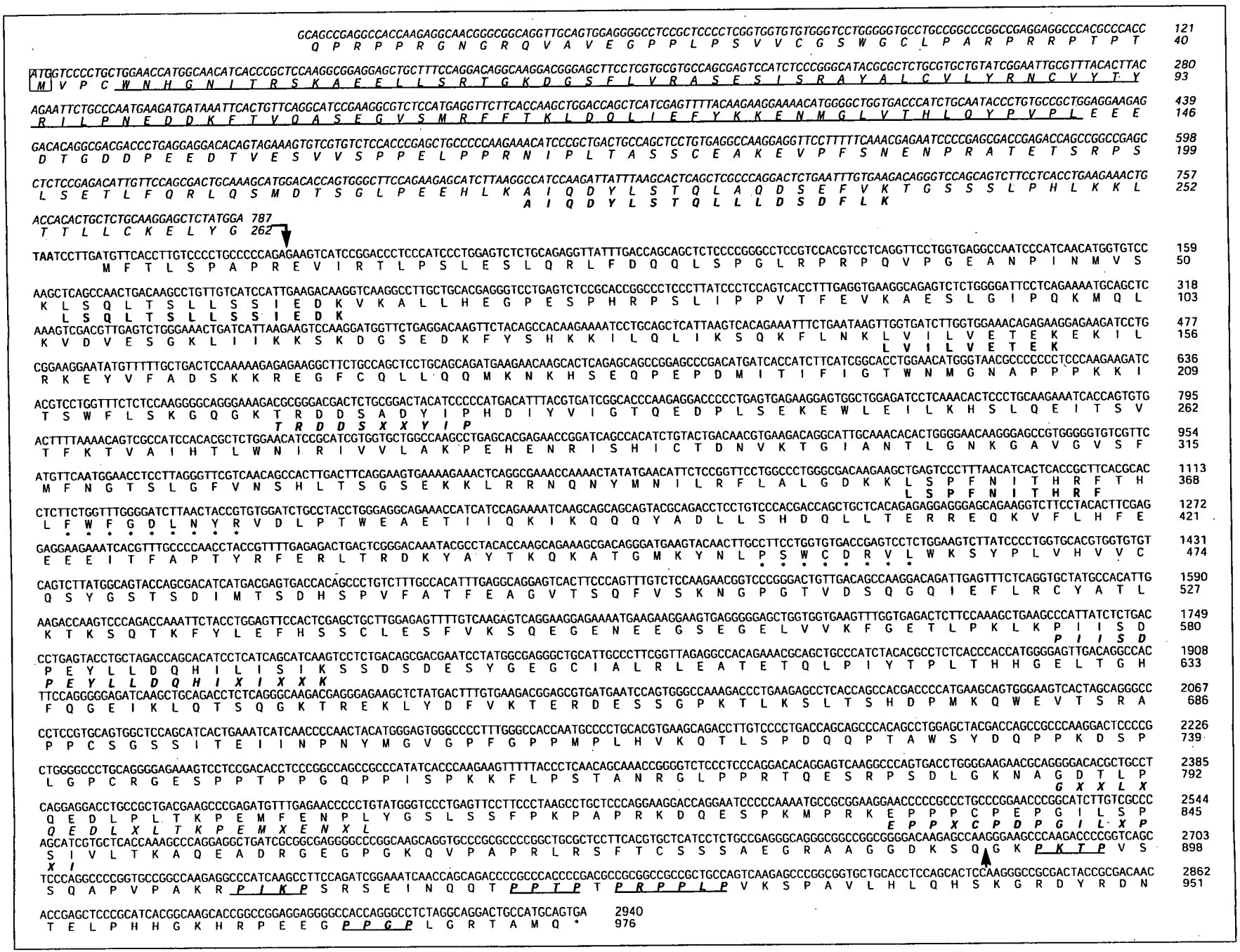
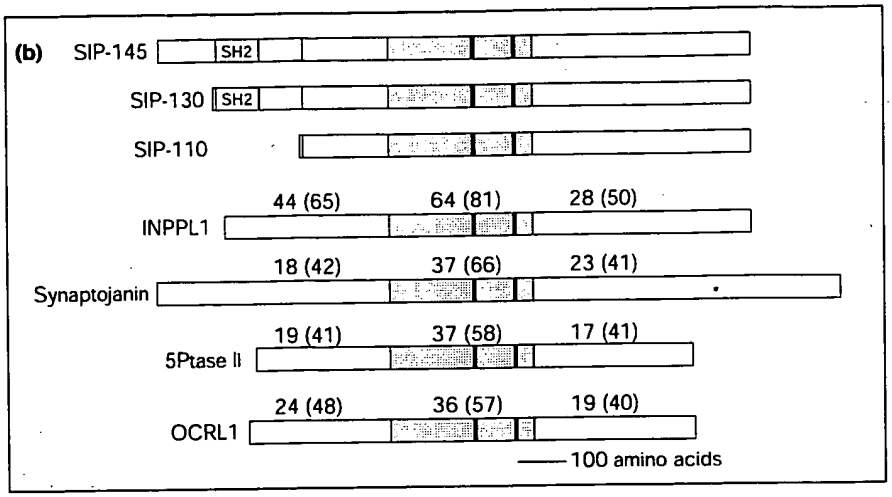


Figure 1 (part (a) is on facing page)



Sequence analysis of SIP-110, SIP-130 and SIP-145, and comparison with inositol polyphosphate 5-phosphatases. **(a)** cDNA and predicted amino-acid sequences of SIP-110 (GenBank accession number U50040) and a partial SIP-145 clone (GenBank accession number U50041). The sequences that are present in SIP-145 but not in SIP-110 are italicized and are numbered separately from the full-length SIP-110 cDNA. The junction between the SIP-145 and SIP-110 sequences is indicated by the large arrow. The putative internal translation start site for SIP-130 is indicated by a box. The predicted amino-acid sequences of SIP-145 and SIP-110 are compared with peptide sequences obtained from purified p145 (bold italics) and p130 (bold type) proteins; X denotes an undetermined residue. The in-frame stop codon 5′ to SIP-110 ATG is in bold type. Proline-rich SH3-binding motifs are indicated by bold underlined italics. The sequence of the partial λgt11 clone of SIP-110 which binds to Grb2 (see text) begins at nucleotide 2679 of the full-length SIP-110 (arrow). The SH2 domain of SIP-145 is underlined. Asterisks denote the conserved sequence motifs that define the inositol polyphosphate 5-phosphatase family [24]. **(b)** Homology between SIP-110, SIP-130 and SIP-145 and other inositol polyphosphate 5-phosphatases. SIP amino-acid sequences were compared to OCRL1 (the protein defective in Lowe's oculocerebrorenal syndrome) [16], type II inositol polyphosphate 5-phosphatase (5Ptase II) [24], INPPL1 [18] and synaptojanin [42]. Percent identity and similarity (parentheses) for the indicated regions are shown. Black boxes indicate the position of the conserved inositol polyphosphate 5-phosphatase sequence motifs (see asterisks in (a)). The asterisk in synaptojanin indicates the stop codon for the 145 kDa form.

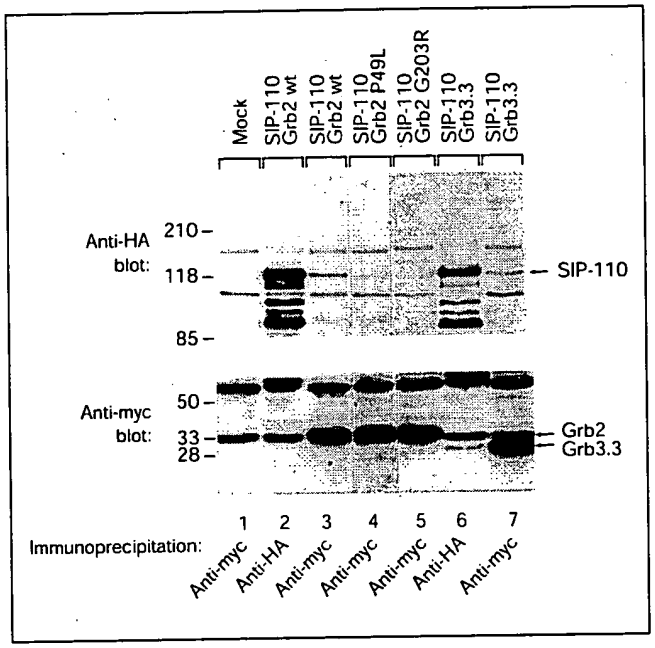
Five p145 peptides and three p130 peptides were sequenced. Surprisingly, four of the p145 peptides and all three p130 peptides contained sequences identical to the 110 kDa SIP-110 (Fig. 1a). One p145-derived peptide contained an aspartic acid residue instead of the glutamic acid at position 839 of SIP-110. One p145-derived peptide contained unique sequence not found in sequence databases (see below and Fig. 1a). The p130 and p145 peptides were identical to widely separated portions of the SIP-110 sequence, suggesting that p130, p145 and SIP-110 are homologous over a large portion of the SIP-110 sequence. These results demonstrated that p130 and p145 have a high degree of similarity to SIP-110, and are likely to be inositol polyphosphate 5-phosphatases. Furthermore, the presence of unique sequence in p145 and the difference in molecular weights of SIP-110 and p145 and p130 demonstrate that they are not the same protein. We have therefore named these proteins SIP-145 and SIP-130 (for signaling inositol polyphosphate 5-phosphatases of 145 kDa and 130 kDa).

We obtained the SIP-145 cDNAs by probing a human lung cDNA library with nucleotide sequences corresponding to SIP-110. A 3537 bp partial cDNA of SIP-145 was obtained with an open reading frame of 1178 amino acids (Fig. 1a). The predicted amino-acid sequence of this cDNA contained all of the peptide sequences isolated from the purified p145 and p130 proteins (Fig. 1a). In one p145-derived peptide, four out of eighteen residues differed from the corresponding predicted amino-acid sequence deduced from the SIP-145 cDNA. Three of these residues represented conservative substitutions. This probably reflects species differences between the peptide, which was derived from mouse p145 protein, and the cDNA encoding SIP-145, which was obtained from a human cDNA library.

Nucleotides 788–3537 of the SIP-145 partial cDNA were identical to nucleotides 37–2786 of SIP-110 (Fig. 1a). This suggested that SIP-110 is a splice variant of SIP-145 which lacks the amino-terminal region of SIP-145. The presence of at least two bands on a northern blot probed with SIP-110 DNA supported this idea (data not shown). Furthermore, an in-frame ATG codon between nucleotides 122–124 of the SIP-145 cDNA was flanked by a consensus translation initiation sequence [22]. Translation beginning at this site would produce a protein of approximate predicted molecular weight of 133 kDa, suggesting that the translation of SIP-130 is initiated at an internal ATG within the SIP-145 mRNA.

The predicted amino-acid sequence of SIP-145 contained an SH2 domain in the amino-terminal region which would be predicted to be present in SIP-130, but is not present in the SIP-110 cDNA (Fig. 1a,b). The 3′ end of the SIP-145 cDNA is identical to SIP-110, and therefore contains several of the proline-rich sequences present in the carboxyl terminus of SIP-110. Because the SIP-145 cDNA was incomplete at the 3′ end, we cannot yet determine if the SIP-145 and SIP-110 are completely identical

Figure 2



SIP-110 associates with the SH3 domains of Grb2 in COS cells. COS7 cells were transiently co-transfected with SIP-110 tagged with the HA epitope and with the following derivatives of Grb2 tagged with the myc epitope: wild-type Grb2 (Grb2 wt), Grb3.3 (containing an inactivating deletion in the SH2 domain), and Grb2 P49L and G203R (containing inactivating point mutations in each of the SH3 domains). Cell lysates were immunoprecipitated with the anti-myc antibody 9E10 (lanes 1, 3, 4, 5 and 7) or the anti-HA antibody 12CA5 (lanes 2 and 6) and immunoblotted with the same anti-HA (upper panel) and anti-myc (lower panel) antibodies. 'Mock' represents the untransfected COS cell background in anti-myc immunoprecipitates. Grb2 migrates with immunoglobulin light chain and is obscured in these gels, whereas the smaller Grb3.3 migrates slightly faster and can be identified (lane 6, lower panel).

in this region. However, the presence of both a SH2 domain and proline-rich sequences in the SIP-145 sequence strongly implicate SIP-145 as an important signaling molecule.

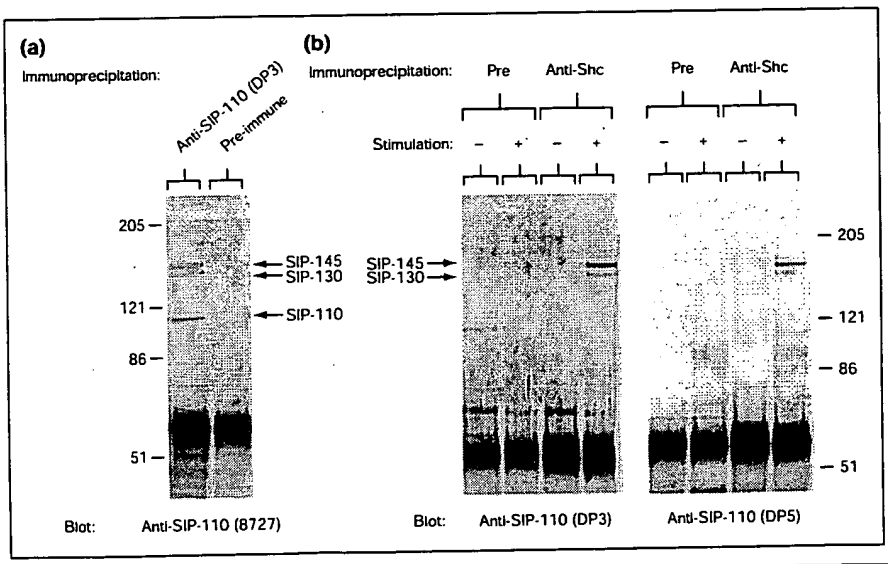
SIP-130 and SIP-145 associate with Shc *in vivo* in response to B-cell activation

The 110 kDa, 130 kDa and 145 kDa SIPs were found to be present in different complexes in cells. Antibodies were raised against three different epitopes of SIP-110. In lysates (data not shown) and in anti-SIP-110 immunoprecipitates (Fig. 3a) from B-cell extracts these antibodies recognized SIP-110, as well as SIP-130 and SIP-145. Shc immunoprecipitates from unstimulated and stimulated BAL 17 cells were analyzed by immunoblotting with these anti-SIP-110 antibodies. All three anti-SIP-110 antibodies specifically recognized proteins of 130 and 145 kDa in Shc immunoprecipitates from stimulated cells, but not in immunoprecipitates from unstimulated cells or in pre-immune immunoprecipitates (Fig. 3b). Taken together, these data demonstrate that the 130 kDa and 145 kDa proteins that were purified are structurally and immunologically similar, but not identical, to SIP-110. Furthermore, the interaction of SIP-130 and SIP-145 with Shc is likely to be important, because SIP-130 and SIP-145 bind Shc only in response to stimulation of cells. SIP-110 was not detected in Shc immunoprecipitates (Fig. 3b), despite the presence of SIP-110 in anti-SIP-110 immunoprecipitates (Fig. 3a). These data suggest that SIP-110 does not form a stable complex with Shc.

Inositol polyphosphate 5-phosphatase activities of SIP-110, SIP-130 and SIP-145.

We next analyzed the enzymatic activity of SIP-110, SIP-130 and SIP-145. Inositol polyphosphate 5-phosphatases

Figure 3

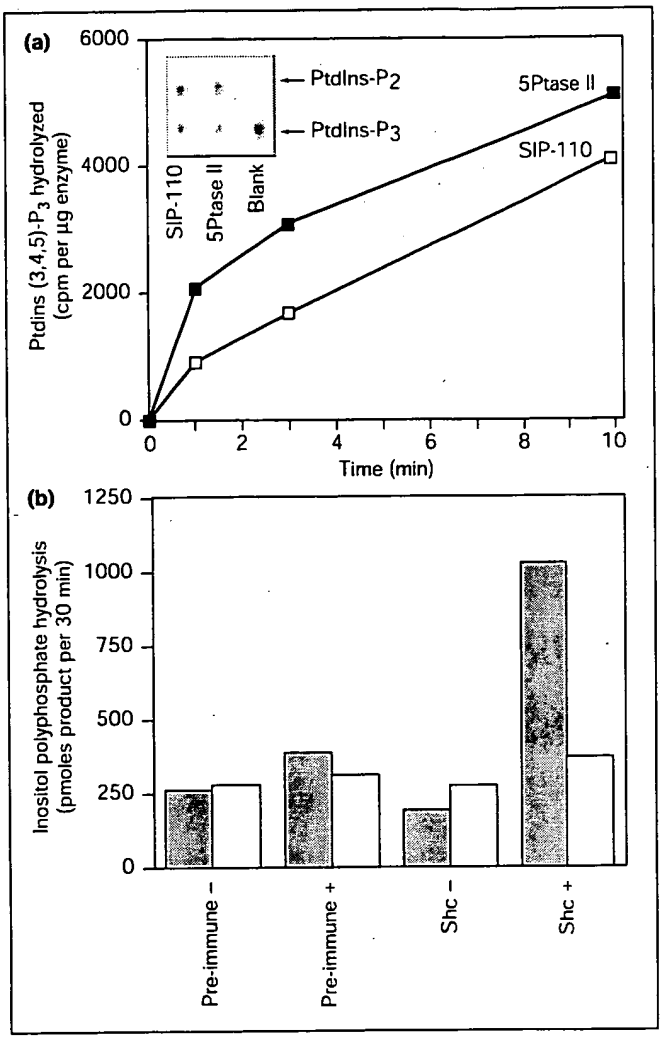


(a) Immunoblotting of SIP-110, SIP-130 and SIP-145. Proteins were immunoprecipitated from B-cell extracts with pre-immune sera, or the anti-SIP-110 antisera DP3, and immunoblotted with the anti-SIP-110 antisera 8727. The positions of SIP-110, SIP-130 and SIP-145 are indicated. **(b)** SIP-130 and SIP-145 specifically associate with Shc in response to B-cell activation *in vivo*. Pre-immune and anti-Shc immunoprecipitates from unstimulated and activated B cells were immunoblotted with two different antibodies raised against separate epitopes of SIP-110 (DP3, left panel; DP5, right panel). The positions of SIP-130 and SIP-145 are indicated by arrows: these bands were absent when immunoblotting was carried out using pre-immune sera (data not shown).

hydrolyze the 5-phosphate from inositol (1,4,5) trisphosphate (Ins(1,4,5)-P_3) and Ins(1,3,4,5)-P_4 [17,19,20,23,24]. A subset of these enzymes can remove the 5-phosphate from phosphatidylinositol (PtdIns) polyphosphates [23–26]. Recombinant SIP-110 hydrolyzed PtdIns (3,4,5)-P_3 to PtdIns (3,4)-P_2, as did a different member of this family of enzymes, 5Ptase II [24] (Fig. 4a). The product of this reaction was shown to be PtdIns (3,4)-P_2 by incubating the product with purified inositol 4-phosphatase, which generated radioactive PtdIns (3)-P (data not shown, and [27]). SIP-110 also hydrolyzed Ins (1,3,4,5)-P_4 to Ins (1,3,4)-P_3 with a K_m of 15.7 μM and Vmax of 100 nmol min^{-1} mg^{-1}. The product of this reaction was Ins (1,3,4)-P_3, because it could be further converted to an Ins-P_2 by purified inositol polyphosphate 1- or 4-phosphatase, neither of which hydrolyze Ins (1,4,5)-P_3 [28,29]. Immunoprecipitation of baculovirus-expressed HA-tagged SIP-110 with anti-HA antibodies also shifted the Ins-P_4-hydrolyzing activity from the supernatant to the protein A–Sepharose pellet (data not shown). This clearly demonstrates that the hydrolyzing activity resides in the SIP-110 protein. The substrate specificity of SIP-110 is different from that of 5Ptase II because SIP-110 did not hydrolyze Ins (1,4,5)-P_3 or PtdIns (4,5)-P_2, both of which are excellent substrates for 5Ptase II (data not shown). Therefore, SIP-110 is an unique inositol and phosphatidylinositol polyphosphate 5-phosphatase which prefers substrates phosphorylated at the 3 position. Because the activity of phosphatidylinositol 3-kinase on PtdIns (4,5)-P_2 would create an ideal substrate for SIP-110, it is possible that these enzymes act sequentially.

To determine whether SIP-130 and SIP-145 are also functional phosphatidylinositol or inositol polyphosphate phosphatases, anti-Shc immunoprecipitates were assayed for their ability to hydrolyze inositol polyphosphates. An activity that hydrolyzed Ins (1,3,4,5)-P_4 was detected in immunoprecipitates of Shc from activated B cells but not in immunoprecipitates from unstimulated cells, or in pre-immune immunoprecipitates (Fig. 4b). No hydrolysis of Ins (1,4,5)-P_3 over background was detected in any of these samples. Further, Shc immunoprecipitates from activated B-cell lysates contained an activity that hydrolyzed PtdIns (3,4,5)-P_3 to PtdIns-P_2 (data not shown). This activity was not observed in Shc immunoprecipitates from unstimulated lysates or in pre-immune immunoprecipitates. Therefore, PtdIns (3,4,5)-P_3 and Ins (1,3,4,5)-P_4 5-phosphatase activities specifically associate with Shc in response to B-cell activation. Like SIP-110, these activities exhibit a preference for 3-phosphorylated substrates. This activity is unlikely to be due to the presence of SIP-110, as no 110 kDa protein was detected in these immunoprecipitates when immunoblotted with anti-SIP-110 antibodies, despite the presence of easily detected SIP-110 in lysates from these cells (Fig. 3). Therefore, one or both of SIP-130 and SIP-145 are also functional inositol polyphosphate phosphatases with a preference for 3-phosphorylated substrates.

Figure 4



Enzymatic activity of SIP-110, SIP-130 and SIP-145. **(a)** Time course of hydrolysis of PtdIns (3,4,5)-P_3 by SIP-110 and 5Ptase II. Production of [^{32}P]PtdIns-P_2 from [^{32}P]PtdIns(3,4,5)-P_3 is plotted as a function of time. Each point is the average of quadruplicate assays. The inset shows an autoradiogram of a thin layer chromatographic (TLC) plate showing conversion of [^{32}P]PtdIns(3,4,5)-P_3 to [^{32}P]PtdIns-P_2 by SIP-110 and 5Ptase II. **(b)** Association of Ins (1,3,4,5)-P_4 phosphatase activity with Shc in response to B-cell activation. Extracts from unstimulated B cells (–) or activated B cells (+) were immunoprecipitated with pre-immune sera or antibodies to Shc (Shc) and assayed for hydrolysis of [^{3}H]Ins(1,3,4,5)-P_4 (gray bars) or [^{3}H]-Ins(1,4,5)-P_3 (white bars). Results are expressed as pmoles of hydrolysis product per 30 min per immunoprecipitate. A blank sample containing no proteins was assayed and subtracted as background. A representative experiment is shown.

Conclusions

These results strongly implicate the inositol polyphosphate 5-phosphatases in Shc- and Grb2-mediated signal

transduction. Three different members of this family of proteins were identified by independent methods to bind to Shc or to Grb2. The three proteins are likely to be products of the same gene. The data are consistent with a model in which activation of tyrosine kinases in cells leads to tyrosine phosphorylation of SIP-130 and SIP-145, which then bind to the PTB domain of Shc. The identification of a related 110 kDa inositol polyphosphate 5-phosphatase which binds to the SH3 domains of Grb2 identifies the inositol polyphosphate 5-phosphatases as a new family of molecules involved in tyrosine kinase signaling. The importance of inositol polyphosphate 5-phosphatases in signaling is strongly suggested by the finding that two signaling molecules known to be important in mediating signaling from tyrosine kinases, Shc and Grb2, bind to similar but distinct inositol polyphosphate 5-phosphatases. Furthermore, SIP-130 and SIP-145 contain a SH2 domain, and are therefore likely to associate with other tyrosine-phosphorylated protein(s). Finally, the preference of these enzymes for inositol substrates phosphorylated at the 3- position suggests that signaling through Shc, Grb2 and phosphatidylinositol 3-kinase are linked. The observation that Shc, Grb2 and phosphatidylinositol-3 kinase can all signal through Ras [5–7,30] suggests that the inositol polyphosphate 5-phosphatases act within a signaling complex to effect Ras-dependent signaling pathways.

Materials and methods

Expression cloning of SIP-110

Human Grb2 cDNA was obtained by screening a human placental library with the *sem5* cDNA of *Caenorhabditis elegans* (a gift from M. Stern, Yale University). Grb2 was expressed in bacteria using pGexKT [31], with the HMK phosphorylation sequence inserted between GST and Grb2. The protein was purified by glutathione–agarose affinity chromatography and labeled with [^{32}P]ATP and HMK to a specific activity of 1×10^7 [$^{32}PO_4$] cpm per μg Grb2 as described [10,15]. The radioactively labeled protein was used to screen a Clonetech oligo dT-primed human placental cDNA expression library [15]. A full-length cDNA clone encoding SIP-110 was obtained by screening a λgt10 human placental cDNA library with the DNA of the original clone (Fig. 1, arrow at position 2679) [32]. Radioactive protein probes were prepared from Vav (amino acids 648–844 [33]), Nck (amino acids 1–149 [34]), and the 85 kDa subunit of phosphatidylinositol 3-kinase (amino acids 1–81 [35]) in a similar manner, and used to test binding to a purified SIP-110-expressing phage under conditions identical to the screening.

Expression of SIP-110 and Grb2 variants in cells

COS7 cells were transiently transfected by the method of Gorman [36]. Full-length SIP-110 with an amino-terminal HA epitope was expressed from the plasmid pCG [37]. Full-length Grb2 was expressed from pCG with a Myc epitope at its carboxyl terminus. Grb3.3, which contains an inactivating deletion in its SH2 domain[21] and a carboxy-terminal Myc epitope tag was generated by PCR and cloned into pCG. Grb2 variants, containing inactivating point mutations in the SH3 domains (human counterparts of natural *C. elegans Sem-5* mutations [8,38]) and a carboxy-terminal Myc epitope tag were also generated by PCR and cloned into pCG. Baculovirus–SIP-110 generated from the plasmid pV-IKS [10] was expressed in Sf9 cells and lysates assayed for inositol polyphosphate 5-phosphatase activity as described below.

Purification of proteins binding to the Shc PTB domain

The p130 and p145 proteins were purified from extracts of BAL 17 B cells stimulated by crosslinking the antigen receptor with anti-immunoglobulin M antibodies as described [39]. Briefly, viable cells were resuspended to 1×10^8 cells ml^{-1} in PBS, incubated at 37 °C for 5 min, sodium orthovanadate was added to 250 μM, and the cells incubated another 5 min at 37 °C. Anti-IgM immunoglobulin (Jackson ImmunoResearch) was added to 1.0 mg per 10^9 cells for 2 min at 37 °C, the slurry was diluted immediately in ice-cold PBS and centrifuged at 4500 × *g* at 4 °C for 5 min. Cell pellets were Dounce homogenized in 20 mM Tris (pH 8.0), 0.5 mM vanadate, and protease inhibitors. The supernatants were loaded at 0.8 ml min^{-1} onto a 15 ml (1.0 cm × 1.3 cm) anti-phosphotyrosine antibody affinity column and eluted at 0.13 ml min^{-1} in the same buffer containing 100 mM phenylphosphate. Column fractions (0.5 ml) were assayed for the p130 and p145 PTB domain-binding proteins as described [10]. Briefly, aliquots of each fraction were analyzed by SDS–PAGE, transferred to nitrocellulose and incubated with ^{32}P-labeled Shc PTB domain protein as a probe. The filters were then washed and exposed to X-ray film. Fractions containing p130 and p145 were then dialyzed against 20 mM Tris–HCl (pH 7.5), 2 mM Na Tungstate, 2 mM Na Arsenate, 1 mM benzamidine and 1 mM DTT, loaded at 1 ml min^{-1} onto a 5 ml Pharmacia Q FF anion exchange column, and eluted with a 100 ml, 0 to 1 M NaCl gradient (1 ml min^{-1}). Peak fractions (2 ml each) eluted in a broad peak between approximately 260 and 330 mM NaCl. These were pooled and analyzed by SDS–PAGE. The 130 kDa and 145 kDa bands were then excised from the gel, digested *in situ* with endoproteinase Lys-C, the peptides purified by HPLC, and sequenced by standard methods [40]. Approximately 50 pmoles of p145 was purified from 4.4×10^{11} cells.

Immunoprecipitations and immunoblotting

BAL 17 cells were stimulated and lysed as described [10,39]. Rabbit antisera was raised against SIP-110 residues 48–231 (antibody DP3), residues 232–500 (antibody DP5) and residues 891–976 (antibody 8727) and against Shc residues 366–473. Immunoprecipitations and immunoblotting were performed as described [10].

Enzyme assays

[^{32}P]PtdIns (3,4,5)-P_3 was prepared as described [27] using PtdIns (4,5)-P_2 and recombinant phosphatidylinositol 3-kinase [30]. TLC-purified [^{32}P]PtdIns (3,4,5)-P_3 (84 000 cpm) was evaporated under nitrogen with 600 μg phosphatidylserine and resuspended into vesicles. Reaction mixtures (25 μl) contained 20 ng baculovirus-expressed recombinant SIP-110 or 31 ng recombinant 5-ptase II [24], 1400 cpm [^{32}P]PtdIns (3,4,5)-P_3, 50 mM Tris (pH 7.5) and 10 mM $MgCl_2$, and were incubated at 37 °C for 1, 3 or 10 min. Hydrolysis was determined as described [27]. Hydrolysis of [^{3}H]Ins (1,3,4,5)-P_4 and [^{3}H]Ins (1,4,5)-P_3 was measured as described [41].

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